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Nucleotide modifications in messenger RNA and their role in development and disease

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Abstract

Modified nucleotides in messenger RNA (mRNA) have been discovered over 40 years ago, but until recently little was known about which transcripts contain them and what their function is. High-throughput sequencing approaches revealed a dynamic landscape of the "Epitranscriptome" for many mRNA modifications in various organisms from yeast to humans. Meanwhile, also many genes encoding mRNA modifying enzymes and auxiliary proteins have been identified yielding functional insights by reverse genetics into their role in development and disease.

Abbreviations: A: adenosine, I: inosine, m6A: *N*6-methyladenosine, m1A: *N*1-methyladenosine, m5C: 5-methylcytosine, hm5C: 5-hydroxymethylcytosine

To date over 100 different nucleotide modifications have been identified in RNA (see: http://modomics.genesilico.pl/ and http://mods.rna.albany.edu). Most of these modifications are present in structural RNAs (e.g. tRNAs and rRNAs), but a few are also present in messenger RNAs (mRNAs) likely affecting all aspects of gene expression [1]. The most prominent modifications in mRNAs are inosine (I), pseudouridine (Ψ), *N*6-methyladenosine (m6A), *N*1-methyladenosine (m1A), 5-methylcytosine (m5C) and 5-hydroxymethylcytosine (hm5C, Figure 1). Here we recapitulate our current knowledge about the genes directing these modifications in humans and model organisms (Table 1) and what their roles are in development and disease (Table 2).

Inosine

The most common modification in mRNA is inosine (I) resulting from hydrolytic deamination of adenine (A) catalyzed by ADARs (Adenosine Deaminases Acting on RNA), which occurs in double stranded RNA (Figure 1). This process is called A-to-I editing and changes the coding because inosine behaves as guanosine and thus base-pairs with cytidine [2]. In protein-coding sequences, A-to-I editing is promoted by the formation of an imperfect fold-back RNA structure between the exonic region containing the editing site and the so-called editing-site complementary sequence (ECS), which is mainly found in intronic regions [3]. Therefore, it has been suggested that RNA editing predominantly occurs before splicing. In addition, ADAR2 edits its own transcript to introduce a new 3' splice site, which in turn results in ADAR2 truncation [1].

Vertebrates have three *adar* genes but one, *adar3,* appears to encode a catalytically inactive protein [2]. Common features of ADAR proteins are a conserved C-terminal catalytic domain, a

nuclear localization signal and up to three distinct dsRBDs (double-stranded RNA-binding domains). Whilst *adar1* and *adar2* are widely expressed, a*dar3* expression is restricted to the brain. The *D. melanogaster* genome encodes a single *adar2*-like gene (*Adar*) [4], while *C. elegans* has two distinct genes [5] and their loss results in neurological phenotypes. On the contrary, ADAR encoding genes are absent in plants, yeasts and filamentous fungi (Table 1) [6].

Historically, A-to-I editing had been found in transcripts of a few ion channel genes and has since expanded to about 80 protein-coding genes [2]. Although lethality of *adar2* mutant mice suffering from epileptic seizures can be rescued by introducing the Q/R edited cDNA of the glutamate receptor GluR2 (GRIA2), recoding for example in AZIN1, COG3 and GRIA2 is functionally important [2, 7]. Since loss of *adar1* in mice leads to aberrant activation of interferon signaling triggered by double-stranded RNA, it is thought that an important function of editing lies also in the immune system.

Recent deep-sequencing revealed the largest number of inosines (approximately 15,000 in human genome) in non-coding repetitive regions, mainly in *Alu* elements, in 5′ and 3′ untranslated regions (UTRs) and *long interspersed elements* (*LINE*) [8]. Herein, the presence of inversely repetitive regions allows the formation of fold-back structures, which can be recognized by the dsRBDs of ADAR enzymes.

One of the most interesting aspects of RNA editing is that I:U mismatch base pairs are less stable than A:U pair [9], therefore the deamination of adenosine in these duplexes destabilizes their overall structure. This feature of RNA editing appears to play an essential role in preventing an aberrant immune response in the presence of endogenous dsRNA that may otherwise trigger the interferon response. Accordingly, these I:U mismatches escape recognition by RIG-I (retinoic

acid-inducible gene I)-like cytoplasmic viral sensor proteins and thus protect cells from an aberrant interferon response [10].

Mutations that compromise A to I editing are linked to several disease phenotypes. The Aicardi-Goutières syndrome (AGS), most frequently characterized by autosomal-recessive inherited mutations in *adar1* gene, manifests as a childhood inflammatory disorder with aberrant interferon expression [11]. Another genetic disorder associated with *adar1* is the rare autosomal-dominant pigmentary genodermatosis dyschromatosis symmetrica hereditaria (DSH) disorder characterized by a mixture of hyper- and hypo-pigmented macules on hands and feet [12].

Hypo-editing of glutamate receptor GluR2 pre-mRNA by downregulated ADAR2 activity has also been associated with amyotrophic lateral sclerosis (ALS) and human glioma [13, 14]. Editing in the serotonin receptor 5-HT_{2c}R pre-mRNA has been linked to various psychiatric disorders including anxiety, bipolar disorder, autism, depression and schizophrenia [2].

In addition, dysregulation of both ADAR1 and/or ADAR2 editing in protein coding genes or miRNAs has also been linked to various cancers including hepatocellular carcinoma, chronic myeloid leukemia squamous cell carcinoma and glioblastoma [15-19]. Further to this, a global analysis of A to I editing revealed altered profiles in many cancers and indicates that editing selectively affects susceptibility to drug treatment [7].

Pseudouridine

Pseudouridine (Ψ) represents the most abundant chemical modification detected in structural RNAs, but is also present in mRNAs. Ψ derives from enzymatic isomerization of uridine by rotation of the nitrogen at the C6 position (Figure 1) [20]. Pseudouridylation can be catalyzed through two different mechanisms: RNA-dependent and RNA-independent [21]. The former

process is carried out by RNA-protein complexes, consisting of a box H/ACA snoRNA, which guides four proteins including the putative rRNA pseudouridine synthase Cbf5 in yeast or DKC1 in humans to introduce the modification site-specifically [21].

The RNA-independent mechanism for pseudouridylation relies on the evolutionary conserved PUS (pseudouridine synthase) domain enzymes (Table 1) [22]. In yeast seven PUS domain containing enzymes are present (PUS1-4, 6,7 and 9), *Drosophila* has 11, while the human genome contains 23 genes with a PUS domain. Recent pseudouridine profiling analyses in yeast and human cells revealed the presence of this modification predominantly in 5'UTRs and in the coding sequence of a few transcripts [23-25]. Further genetic analyses in yeast allowed for assigning individual pseudouridines to each of the seven pseudouridine synthases (PUS1-4, 6,7 and 9), but all of these enzymes modify both mRNAs and structural RNAs [23]. In HEK293 cells most of the Ψ modifications are introduced by PUS1, PUS4 and PUS7 [25].

Interestingly, pseudouridine levels in several mRNAs are increased after heat shock and this involved cytoplasmic translocation of PUS7, highlighting a dynamic program under stress conditions, whereas Cbf5-mediated pseudouridylation is stress-independent. The function of Ψ in mRNA is not known, but might involve alteration of RNA structure, or might alternatively target modified mRNAs to heat shock-induced stress granules [25]. Recently, it has also been shown that Ψ can suppress translational termination if present in either of the three stop codons [26].

Defects in DKC1 (H/ACA ribonucleoprotein complex subunit 4) and PUS1 (tRNA pseudouridine synthase A) have been both associated to human disorders (Table 2). Germline mutations in the human *dkc1* (dyskeratosis congenita 1) gene cause the bone marrow failure disorder dyskeratosis congenital l [27]. Associated with this disease is also poor maintenance of

telomeres due to lower pseudouridine levels in the ncRNA component of the telomerase complex (TERC) [25]. In addition, aberrant expression of *dkc1* has also been linked to cancer predisposition [28, 29].

Noteworthy, various allelic variants of PUS1 show impaired mitochondrial and cytoplasmic tRNA pseudouridylation leading to various condition such as myopathy, lactic acidosis and sideroblastic anemia 1 (MLASA), a rare autosomal recessive oxidative phosphorylation disorder [30].

A role for snoRNA mediated pseudouridylation has been found in serotonin receptor 5-HT_{2c}R alternative splicing regulation contributing to the disease of the Prader-Willi syndrome. These patients do not express the brain-specific C/D box snoRNA HBII52 due to an imprinting defect indicating that this complex involved in pseudouridylation has additional snoRNA guided roles beyond modifying mRNA [31, 32].

_N_6-methyladenosine

The most abundant internal modification in mRNA is *N*6-methyladenosine (m6A, Figure 1) [33, 34]. Although first discovered in the 70s, lack of molecular and genetic tools has hindered identifying its functional relevance until recent anti-m6A antibody pull-down and high-throughput sequencing approaches [35-38]. These efforts revealed transcriptome-wide maps of m6A in humans, plants and yeast, but also indicated a high percentage of false positives due to the limited specificity of anti-m6A antibodies [37-40]. In humans, about 12,000 m6A sites were mapped on 7000 coding and 300 non-coding genes with an average of 3-5 sites per transcript [37]. Interestingly, m6A preferentially peaks in the 5'UTR and around the stop codon, but is also present in long exons in the consensus sequence RRACH (where R is a purine, and H is

adenosine, cytidine or uracil) [37, 38, 41, 42]. In accordance with their localization in UTRs, presence of m6A has been shown to affect mRNA stability and translation [43-45]. Although m6A is also found in introns, its exact roles in splicing regulation remain to be established.

The proteins governing the dynamic m6A landscape can be divided into three classes: writers, readers, and erasers. Biochemical characterization of the human methylosome, the m6A writer, revealed two complexes of 200 kDa and 875 kDa containing the catalytic activity MT-A encoded by the *mettl3* gene, which places the m6A epimark in single stranded RNA [46]. Recent proteomics and reconstitution assays identified further components including a second catalytic subunit METTL14, the splicing factors WTAP (Wilm's tumor associating protein) and Virilizer as well as a number of other proteins [47-50]. Orthologues of *mettl3* and *mettl14* are found in *Drosophila*, plants and budding yeast, but seem to be absent in worms and fission yeast [50, 51]. Loss of *mettl3* results in embryonic lethality in mice and plants, while in *S. cerevisiae*, expression of the *mettl3* orthologue *inducer of meiosis4* (*ime4*) and consequently m6A are found only during meiosis [50, 52, 53]. Other functions include roles in mammalian embryonic stem cell renewal, the circadian clock and cap independent translation for directing the heat-shock response [52, 54-57].

Readers of the m6A epimark belong to the YT521-B homology (YTH) protein family. The YTH domain has a fifty fold higher affinity towards m6A compared to unmodified adenosine. Humans have five YTH proteins (YTHDF1-3 and YTHDC1 and 2), *Drosophila* has two (CG6422 and YT521-B) and *S. cerevisiae* has one (Mrb1), while plants have thirteen orthologues [33]. Intriguingly, the fission yeast YTH orthologue Mmi1 binds RNA in a different mode than the other YTH proteins consistent with the absence of m6A in mRNA in this organism [58].

While reader proteins directly bind the modified nucleotide, m6A has also been shown to promote RNA structural remodeling to favor recruitment of the RNA binding protein HNRNPC in a stem loop of the MALAT1 non-coding RNA [59].

Eraser proteins are m6A demethylases making this modification completely reversible. Currently two such proteins are known: Fat mass and obesity associated (FTO) and ALKBH5, a member of the Fe2/α-ketoglutarate-dependent dioxygenase family [60, 61].

Whereas *alkbh5* knockout mice have impaired male fertility [62], alterations in FTO expression have been connected to altered energy metabolism leading to obesity, type 2 diabetes, growth retardation, developmental delay, facial dysmorphism and cancer [63-66].

_N_1-methyladenosine

Yet another modification analysed for its genome-wide occurrence is *N*1-methyladenosine (m1A) [67, 68]. Elegantly, m1A can be converted to m6A by the Dimroth reaction and then analysed using the m6A antibody. This analysis revealed a dynamic m1A landscape with a predominant peak near the start codon in human and yeast mRNA in response to cellular stress. Noteworthy, the m1A modification harbours a positive charge under physiological conditions. This may affect the canonical base pairing, altering secondary and tertiary structure, or affect RNA-protein interactions. In contrast to the m6A modification, m1A leads to abortive reverse transcription, which can be employed to validate this modification [69].

While the catalytic entity responsible for installing m1A is still not known, overexpression of the *alkbh3* gene results in decreased m1A levels. This makes ALKBH3 a likely candidate for demethylating m1A in mRNAs [67].

5-methylcytosine and 5-hydroxymethylcytosine

The methyl group in 5-methylcytosine (m5C) prevents the conversion to uracil by bisulfite treatment. Applying this feature in combination with high-throughput sequencing detected a considerable number of m5C in mRNA [70]. In mRNA, m5C is mainly present in untranslated regions in the proximity of Argonaute family protein binding sites suggesting roles in mRNA stability and translation [70].

The only enzyme currently known to methylate cytosine in mRNA is NSUN2 (NOP2/Sun RNA methyltransferase family member 2) [70, 71]. Evolutionary conserved NSUN2 localizes to the nucleus, but in addition to mRNA also methylates cytosines in tRNAs as revealed by a mutant NSUN2 protein tethering itself to the site of methylation (Table 1)[72].

NSUN2 is required for stem cell differentiation and cell proliferation, but has also been linked to human cancers [71, 73-76]. In addition, methylation of the mRNA for intracellular cell adhesion molecule (ICAM-1) promotes inflammation via increased translation [77]. In accordance with NSUN2's increased expression in the brain, mutations have been identified that cause intellectual disability [78].

Although *Drosophila* seems not have m5C DNA methylation, its genome contains a *Ten-eleven-translocation* (*Tet*) orthologue involved in DNA demethylation in vertebrates via hydroxylation of the methylgroup, which is then further modified to 5-formylcytosine and 5-carboxylcytosine leading to glycosylation and removal via DNA excision repair. This paradox is explained by the capacity of human TET3, but not TET1 or TET2, to hydroxylate m5C in RNA [79]. Accordingly, hm5C can be detected in RNA of animals and plants, but at lower levels than m5C [80]. Using an antibody against hm5C and high throughput sequencing 3058 hm5C methylation sites have been detected in 1597 transcripts in *Drosophila* S2 cells [81]. Although these levels

are low, hm5C in mRNA is functionally relevant as *Drosophila tet* mutants die during pupal development and show defects in neuroblast proliferation [81].

Noteworthy, *tet*-null ES cells genetically deprived of all TET enzymes, still show detectable levels of hm5C, suggesting that m5C oxidation can also be catalyzed by different enzymes or induced by cellular reactive oxygen species. Accordingly, the hm5C modification is also detectable in *C. elegans* and *A. thaliana,* which do not have orthologues of TET in their genomes [79].

Conclusions

Various mRNA modifications are present from yeast to humans and the factors installing these modifications are evolutionarily conserved indicating ancient mechanisms to regulate gene expression at the level of the mRNA. Recent high-throughput sequencing approaches have brought about a dynamic landscape for many mRNA modifications initiating the new field of epitranscriptomics. Surely, more mRNA modifications will turn up, but future challenges lie in connecting the dynamic nature of mRNA modifications to regulatory pathways relevant to development and human disease.

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Figure legends

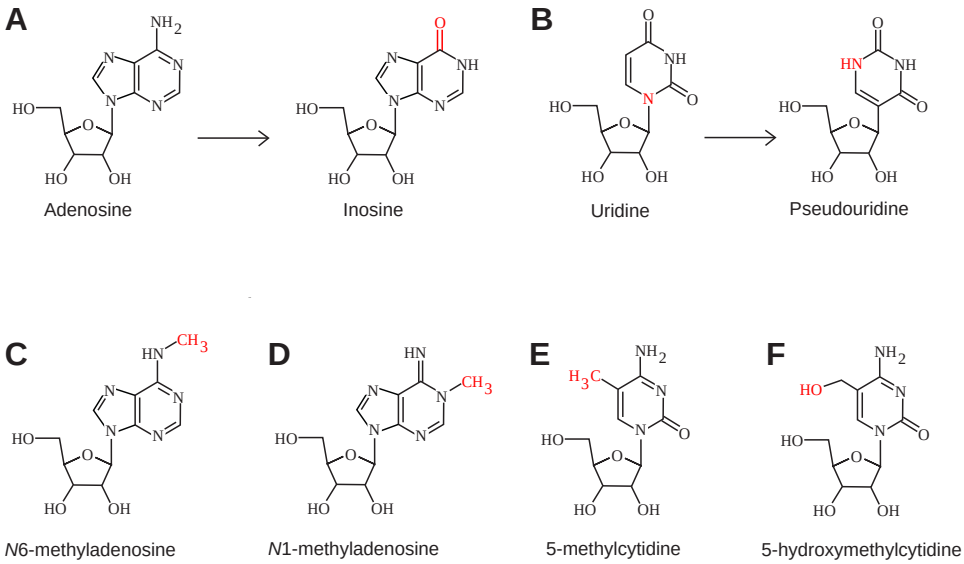
Figure 1 Nucleotide modifications in mRNA. A) Inosine, B) Pseudouridine, C) *N*6-methyladenosine, D) *N*1-methyladenosine, E) 5-methylcytosine and F) 5-hydroxymethylcytosine

Table 1: Orthologues of mRNA modification enzymes in humans, flies, worms, yeast and plants. Orthologues are as annotated in flybase, or searched for in data bases of other organisms (http://flybase.org/, http://www.wormbase.org, https://www.arabidopsis.org/index.jsp and www.yeastgenome.org). n.d.: not determined.

	Orthologues of mRNA modifying enzymes					
	H. sapiens	***D. melanogaster***	***C. elegans***	***S. cerevisiae***	***A. thaliana***	**References**
mRNA modification						
A-to-I editing	ADAR1	absent	ADR-1			
	ADAR2	ADAR	ADR-2	absent	absent	[2, 19]
	ADAR3	absent	absent			
Pseudouridylation	DKC1	NOP60B	K01G5.5	CBF5	AT3G57150	[21, 23-25, 82]
	PUS1	CG4159	PUS1	PUS1		
	PUSL1	CG34140	Y73B6BL.29			
				PUS2		
	PUSD2	RluA-1/-2	K07E8.7			
	PUS3	CG3045	TAG-124	PUS3		
	PUS4	CG7849	Y43B11AR.3	PUS4		
	PUS6			PUS6		
	PUS7	CG6745	B0024.11	PUS7		
	PUS9		PUS9	PUS9		
	PUS10	CG3709	Y48C3A.20			
m6A methylation	METTL3	dIME4	absent	IME4	AT4G10760	[46, 53]
	METTL14	dKAR4	absent	KAR4	AT4G09980	
m1A methylation	unknown	n.d.	n.d.	unknown	n.d.	
m5C methylation	NSUN2	NSUN2	Y48G8AL.5	TRM4	AT2G22400	[70, 80]
hm5C hydroxylation	TET3	dTET	absent	absent	absent	[79-81]

Table 2: Human diseases associated with mRNA modification genes.

mRNA modification	Gene	Human disorder
I	*adar1* (adenosine deaminase, RNA-specific, 1)	Chronic myeloid leukemia (CML)[19] Metastatic melanoma[15] Human hepatocellular carcinoma(HCC)[83] Esophageal squamous cell carcinoma (ESCC)[17] Dyschromatosis symmetrica heriditaria (DSH)[84] Aicardi- Goutieres syndrome (AGS)[11]
	adar2 (adenosine deaminase, RNA-specific, 2)	Glioblastoma multiforme (GBM)[16] Alzheimer disease[85] Amyotrophic lateral sclerosis (ALS)[86]
Ψ	*dkc1* (dyskerin pseudouridine synthase 1)	Diskeratosis congenital[27] Pituitary tumorigenesis[28] Prostate cancer[29]
	pus1 (pseudouridine synthase 1)	Mitocondrial myopathy, lactic acidosis and sideroblastic anemia (MLASA)[30]
m6A	*wtap* (Wilms tumor 1 associated protein)	Hypospadias[87] Acute myelogennous leukemia (AML) [88] Cholangiocarcinoma[89]
	fto (fat mass- and obesity-associated gene)	Obesity[63] Coronary heart disease (CAD)[90] Growth retardation, development delay and facial dysmorfism[64] Type 2 diabetes [65] Cancer[91] [92]
	alkbh5 (alkB homolog 5, RNA demethylase)	Infertility[62] Major depressive disorder (MDD) [93]
m5C	*nsun2* (nol1/nop2/sun domain family, member 2)	Breast cancer [74] Autosomal- recessive intellectual disability [78]


A
B
C
D
E
F
Adenosine
Inosine
Uridine
Pseudouridine
N6-methyladenosine
N1-methyladenosine
5-methylcytidine
5-hydroxymethylcytidine